

Chemical Modification of DNA with Muta-Carcinogens. I. 3-Amino-1-methyl-5H-pyrido[4,3-b]indole and 2-Amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole: Metabolic Activation and Structure of the DNA Adducts

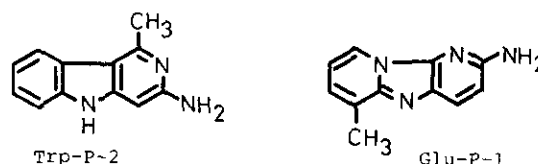
by Yuichi Hashimoto* and Koichi Shudo*

3-Amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) and 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) are potent mutagen/carcinogens isolated from pyrolyzates of tryptophan and glutamic acid, respectively, and they have been found to exist in many cooked foods. Trp-P-2 and Glu-P-1 bind to DNA covalently after metabolic activations. The compounds are oxidized to the corresponding hydroxylamines (N-OH-Trp-P-2 and N-OH-Glu-P-1) by microsomes. N-OH-Trp-P-2 and N-OH-Glu-P-1 are the proximate forms of Trp-P-2 and Glu-P-1, respectively. They are further activated by cytosol to the O-acyl derivatives, which bind covalently with DNA. The structures of the modified nucleic acid bases were identified as 3-(C⁸-guanyl)amino-1-methyl-5H-pyrido[4,3-b]indole (Gua-Trp-P-2) and 2-(C⁸-guanyl)amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Gua-Glu-P-1). These initial events caused by Trp-P-2 and Glu-P-1 were established chemically, both *in vitro* and *in vivo*.

Introduction

The goal of chemical investigation of chemical carcinogenesis is to elucidate the chemical events caused by chemical carcinogens in the body; such knowledge would be useful for the effective prevention and treatment of cancer. A large number of carcinogens is believed to be present in our environment, many of which should be detectable as mutagens by Ames' assay using bacteria and mammalian metabolic enzyme systems (1). An important current problem is the presence of carcinogens in cooked foods. It was shown that cooked foods such as broiled beefsteak, broiled fish, and hamburger contain strong mutagens formed by heating during the cooking process (2-5). These mutagens were found to be formed by thermal reactions of the food components, especially proteins, or amino acids (2-8). Among these compounds, 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) isolated from a pyrolyzate of L-tryptophan (7) and 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole (Glu-P-1) isolated from a pyrolyzate of L-glutamic acid (8) were proved to be carcinogenic (9,10). The structures of these compounds were deduced from spectral data

and X-ray analysis results and finally confirmed by synthesis (11,12). These carcinogens were found to exist in cooked foods such as broiled cuttlefish (13), grilled beef (14), broiled sardine (15), and beef extracts (16). They seem likely to be distributed widely in cooked foods and might play an important role in human carcinogenesis. Quantitative evaluation of the cancer risk due to these carcinogens still requires extensive further chemical, analytical and biological studies.



An important step in carcinogenesis is thought to be the initial attack on the DNA molecule by a chemical or a so-called ultimate carcinogen which is formed metabolically from a parent carcinogen. The covalent binding of the ultimate carcinogen to the DNA bases creates a premutational lesion that is processed by repair, rep-

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Table 1. Binding of [³H]Trp-P-2 to DNA and homopolynucleotides.

DNA or nucleotide	Addition	Bound [³ H]Trp-P-2, $\mu\text{mole/mole P}$
DNA	Microsomes	59
DNA	Microsomes ^a	230
DNA	Heated microsomes	6
Poly A	Microsomes	2
Poly G	Microsomes	42
Poly C	Microsomes	3
Poly U	Microsomes	4

^aThe incubation was repeated three times.

lication, and recombination enzymes *in vivo* and eventually may be converted to a mutation. Elucidation of the structures of DNAs modified with mutagenic carcinogens is essential for an understanding of the molecular basis for alteration of gene expression in mutagenesis. Studies on the chemical structures of DNAs modified with acetylaminofluorene (17), methylaminoazobenzene (18), benzo[a]pyrene (19), 4-nitroquinoline-N-oxide (20), aflatoxin B1 (21), and naphthylamine (22) represent pioneering work in this field. In this article, we review our data on the metabolic activation of Trp-P-2 and Glu-P-1, and on the modification of DNA by these compounds.

Structures of the Modified Nucleic Acid Base

Trp-P-2 and Glu-P-1 show very high mutagenicity toward *Salmonella typhimurium* TA98 only in the presence of microsomal proteins. In accordance with this fact, Trp-P-2 and Glu-P-1 bind to DNA *in vitro* only in the presence of rat liver microsomes (23,24). The covalent binding of these carcinogens with DNA, as well as the expression of their mutagenicity, requires metabolic activation. An experiment on the binding of Trp-P-2 to nucleic acids was performed by the use of radio-

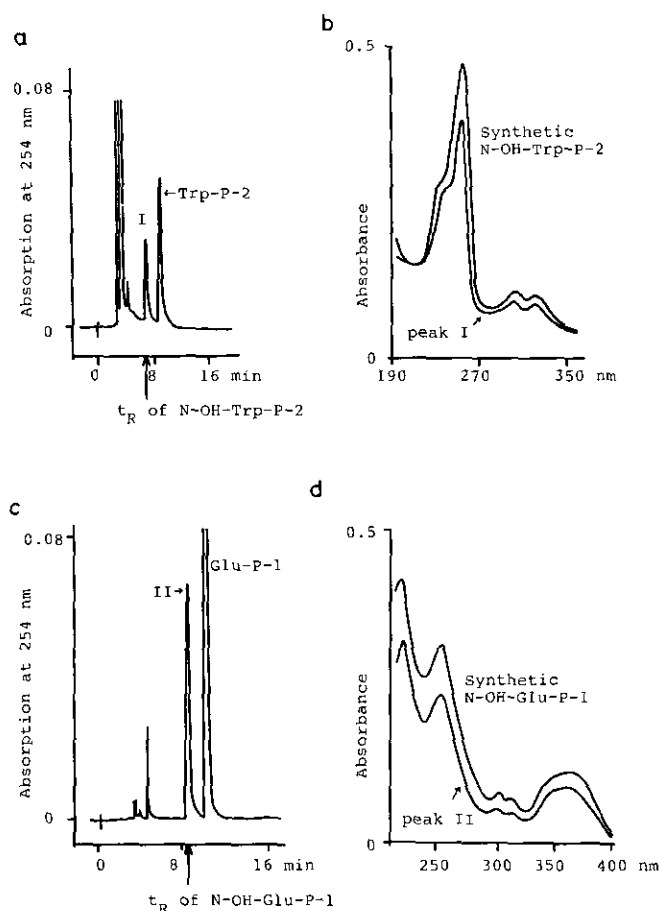
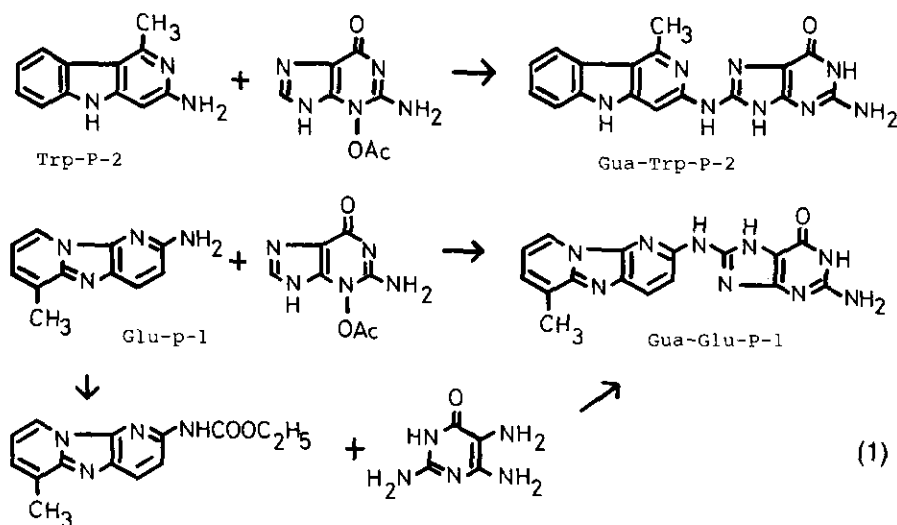
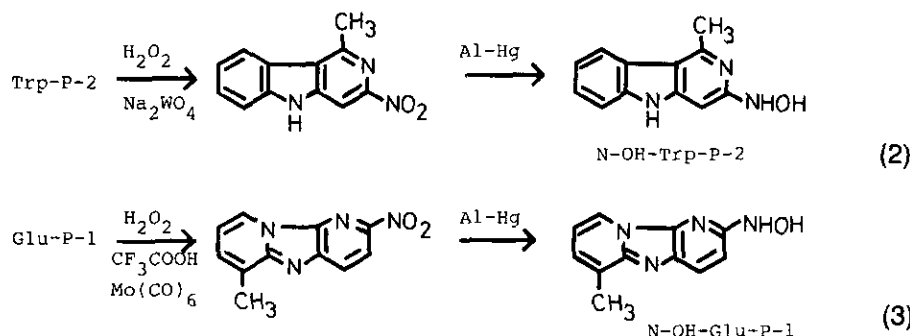


FIGURE 1. Identification of microsomal metabolites of Trp-P-2 and Glu-P-1: (a) HPLC of microsomal metabolites of Trp-P-2 (column: Polygasil₅C₁₈, 4.6 mm ID \times 150 mm, solvent CH₃CN(40%)–0.02M–KH₂PO₄(60%), flow rate 0.7 mL/min); (b) UV spectra of peak I and N-OH-Trp-P-2; (c) HPLC of microsomal metabolites of Glu-P-1 (column: Zorbax SIL 4.6 mm ID \times 250 mm, solvent CH₂Cl₂(75%)–CH₃CN(20%)–CH₃OH(5%), flow rate 1.0 mL/min); (d) UV spectra of peak II and N-OH-Glu-P-1.





active [^3H] Trp-P-2. From the radioactivity, the amount of Trp-P-2 bound to DNA was calculated to be about 60 $\mu\text{mole/mole P}$, and the binding increased to 230 $\mu\text{mole/mole P}$ on three successive incubations of the DNA with microsomes and Trp-P-2 (Table 1) (23). No radioactivity of Trp-P-2 bound to the homopolynucleotides poly A, poly C, and poly U on incubation with or without microsomes. Trp-P-2 bound significantly to poly G in the presence of microsomes. The result of the binding experiment with Glu-P-1 was similar to that in the case of Trp-P-2. These results suggest that Trp-P-2 and Glu-P-1 bind to guanine residues in DNA. The hydrolysis

of DNAs modified with Trp-P-2 and Glu-P-1 gave only one Trp-P-2-modified base and one Glu-P-1-modified base, respectively, and these were purified by high performance liquid chromatography (HPLC) (24,25). The hydrolysis of the Trp-P-2-modified base and Glu-P-1-modified base with aqueous alkali gave 8-hydroxyguanine and the starting amines (Trp-P-2 and Glu-P-1, respectively). The above results and analysis of spectroscopic data of the modified bases suggest that the structures of the modified bases are 3-(C⁸-guanyl)amino-1-methyl-5H-pyrido[4,3-b]indole (Gua-Trp-P-2) and 2-(C⁸-guanyl)amino-6-methyldipyrido(1,2-

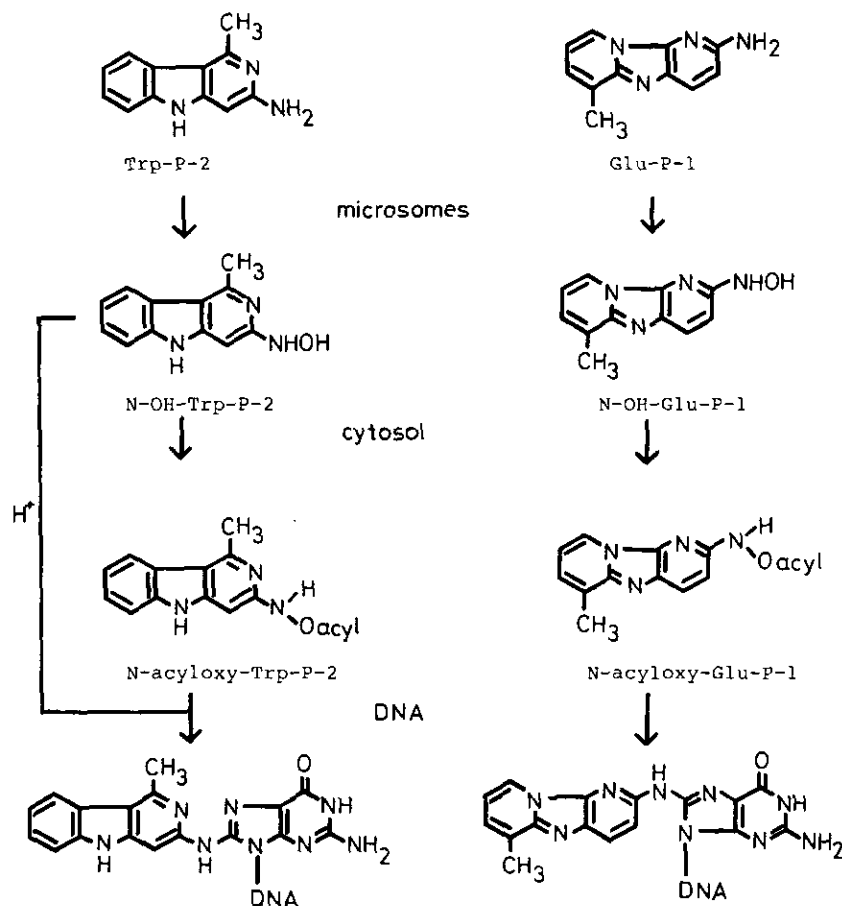


FIGURE 2. Pathways of DNA modification by Trp-P-2 and Glu-P-1.

a:3',2'-d'imidazole (Gua-Glu-P-1). To confirm the proposed structures, Gua-Trp-P-2 and Gua-Glu-P-1 were synthesized as shown in Eq. (1). The synthesis was performed by the nucleophilic substitution of 3-acetoxypurine with Trp-P-2 or Glu-P-1. A ring condensation reaction of a carboethoxy derivative of Glu-P-1 with 2,4,5-triamino-6-hydroxypyrimidine also gave Gua-Glu-P-1 (26,27). The methods may be generally useful to prepare guanines modified with carcinogenic amines at the 8-position, which seems to be a general site of attack by chemical carcinogens. The nucleic acid bases modified with Trp-P-2 and Glu-P-1 *in vitro* were identical with the synthetic Gua-Trp-P-2 and the synthetic Gua-Glu-P-1, respectively (26,27).

Active Metabolites of Trp-P-2 and Glu-P-1

The structures of the modified nucleic acid bases, Gua-Trp-P-2 and Gua-Glu-P-1, suggest that the metabolically activated forms of Trp-P-2 and Glu-P-1 are the corresponding hydroxylamines (N-OH-Trp-P-2 and N-OH-Glu-P-1, respectively), because arylhydroxylamines can be electrophiles after the heterolysis of the N-O bond. In fact, treatment of Trp-P-2 and Glu-P-1 with rat liver microsomes gives N-OH-Trp-P-2 and N-OH-Glu-P-1, respectively, as the major primary metabolites (Fig. 1) (28,29). The structures of these metabolites were confirmed by comparison with the authentic hydroxylamines synthesized as shown in Eqs. (2) and (3). The reactions of N-OH-Trp-P-2 and N-OH-Glu-P-1 with DNA were next investigated. N-OH-Trp-P-2 binds covalently with DNA only under slightly acidic conditions (pH5) to give Gua-Trp-P-2 after hydrolysis of the modified DNA, though in a very low yield ($6 \times 10^{-3}\%$ based on guanine residues in DNA). However, when the hydroxylamine was *O*-acetylated, the resulting *N*-acetoxy-Trp-P-2 (N-OAc-Trp-P-2) showed efficient covalent binding with DNA even under neutral conditions to give Gua-Trp-P-2 in a yield of about 1% from guanine residues in DNA after hydrolysis of the modified DNA (26,28). Gua-Trp-P-2 was the only product (modified nucleic acid base) obtained in the DNA modifications. The efficiency of the modification with N-OAc-Trp-2 is more than 30 times that of the microsome-mediated modification of DNA with Trp-P-2. This suggests that *O*-esterification may be involved in the *in vivo* modification of DNA, though the free hydroxylamine itself must also contribute to the modification to some extent. These results are presented in Figure 2, as a proposed path of DNA modification by Trp-P-2. In accordance with this path, N-OH-Trp-P-2 itself was found to be a direct mutagen toward *Salmonella typhimurium* TA98. However, the addition of cytosol, which contains large amounts of esterifying enzymes, enhances the mutagenicity. The chemistry of DNA modification with Glu-P-1 is slightly different, in contrast. An active metabolite of Glu-P-1, N-OH-Glu-P-1 did not react with DNA even under acidic conditions. It reacted with DNA only

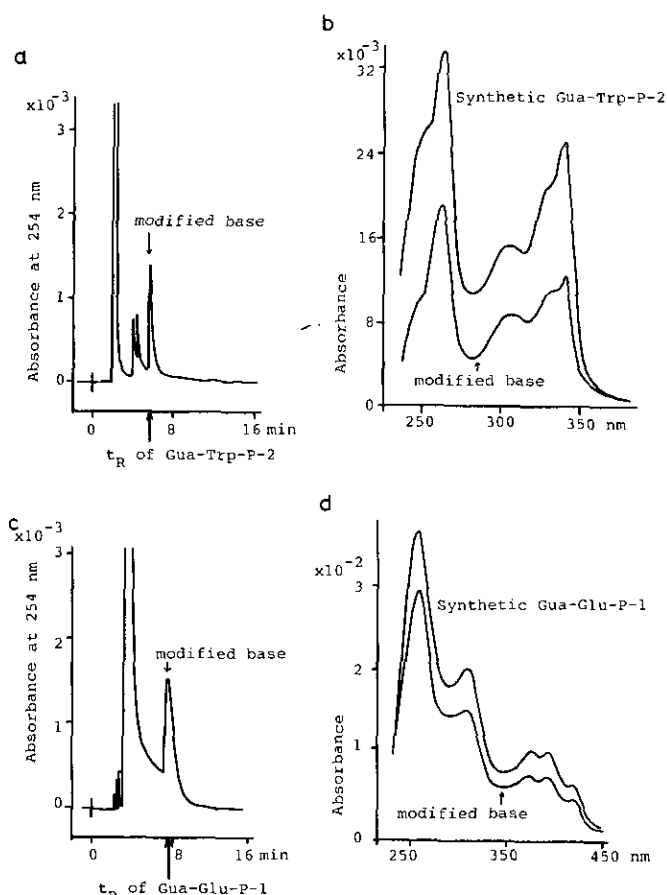


FIGURE 3. Identification of Gua-Trp-P-2 and Gua-Glu-P-1 isolated from liver DNA of rats treated with Trp-P-2 and Glu-P-1: HPLC (a) and UV spectra (b) of Gua-Trp-P-2 liberated from liver DNA of rats treated with Trp-P-2. (column: Polygosil C_{18} 4.6 mm ID \times 250 mm, solvent $\text{CH}_3\text{OH}(40\%)$ - $\text{H}_2\text{O}(59\%)$ -conc. $\text{NH}_4\text{OH}(1\%)$, flow rate 1.0 mL/min); HPLC (c) and UV spectra (d) of Gua-Glu-P-1 liberated from liver DNA of rats treated with Glu-P-1. (column: Polygosil C_{18} 4.6 mm ID \times 250 mm, solvent $\text{CH}_3\text{OH}(35\%)$ - $\text{H}_2\text{O}(64\%)$ -conc. $\text{NH}_4\text{OH}(1\%)$, flow rate 1.0 mL/min).

after esterification under neutral conditions to give Gua-Glu-P-1 in a yield of about 2% from guanine residues in DNA after hydrolysis of the modified DNA (Fig. 2) (27,29). Therefore, N-OH-Glu-P-1 is a so-called proximate form of Glu-P-1, and the ultimate form is an *O*-acetylated derivative of N-OH-Glu-P-1.

Modification of DNA *in Vivo*

The paths of DNA modification with Trp-P-2 and Glu-P-1 *in vitro* were established as mentioned above. It is important to know whether the same modification of DNA by these carcinogens occurs *in vivo*. We found that Trp-P-2 and Glu-P-1 bound to rat liver DNA *in vivo* (30). The structure of the major modified nucleic acid base liberated from liver DNA of rats treated with Trp-P-2 was identified by comparison of its retention time and UV spectrum with those of authentic Gua-Trp-P-2 (Fig. 3). The amounts of bound Trp-P-2 were 2.0×10^{-5} mole/mole P for DNA and 5.0×10^{-6} mole/

mole P for rRNA. The structure of the modified base liberated from liver DNA of rats treated with Glu-P-1 was identified by comparison of its retention time and UV spectrum with those of authentic Gua-Glu-P-1 (Fig. 3). The amounts of Glu-P-1 bound to nucleic acids were 2.5×10^{-5} mole/mole P for DNA and 3.0×10^{-6} mole/mole P for rRNA. The binding of Glu-P-1 was rather stable because roughly the same amount of Gua-Glu-P-1 was found in modified DNA extracted from the liver 48 hr after injection of Glu-P-1.

These findings suggest that injected Trp-P-2 and Glu-P-1 are metabolically activated to reactive metabolites, probably the corresponding hydroxylamine and/or their O-acyl derivatives, and then bind to DNA and rRNA *in vivo*. This work shows that the reactions established chemically as the initial steps of chemical mutagenesis or carcinogenesis (Fig. 2) occur similarly *in vivo*. These pathways *in vivo* might be, at least in part, responsible for the effectiveness of the compounds as mutagen/carcinogens.

Conclusion

The pathways of DNA modification by the mutagenic Trp-P-2 and Glu-P-1 *in vitro* and *in vivo* were established chemically. The reactive ultimate form of Trp-P-2 is N-O-acyl-Trp-P-2, which is far more reactive than N-OH-Trp-P-2. The ultimate form of Glu-P-1 is also N-O-acyl-Glu-P-1 and the proximate form is N-OH-Glu-P-1. The site of modification is the C⁸ position of guanine residues in DNA.

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